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分析拟南芥叶片在激素促进的衰老中内源激素变化来解析抑制磷脂酶 Dα1 延缓激素促进衰老的机制*

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摘要: 采后衰老进程在很大程度上受到内源和外源激素的影响。抑制拟南芥中磷脂酶 Dα1 (phospholipase Dα1, PLDα1) 的表达后, 使得外源脱落酸 (abscisic acid, ABA) 和乙烯加速的离体叶片衰老过程在一定程度上得到了缓解。然而, 内源激素在这个过程中的作用尚不清楚。本研究对比分析了野生型和 PLDα1 缺失型两种基因型拟南芥叶片在 3 种不同人工老化过程中 (离体诱导的、外源 ABA 和乙烯促进的衰老过程), 内源 ABA, 茉莉酸甲酯 (methyl jasmonate, MeJA)、吲哚乙酸 (indole-3-acetic acid, IAA)、玉米素核苷 (zeatin riboside, ZR) 和赤霉素 (gibberellic acid, GA_3) 的含量变化。这 5 种激素对 3 种不同衰老处理方式的响应模式表明了人工老化过程存在着两个不同阶段, 并且在衰老早期每种激素的变化模式相同。PLDα1 功能缺失使得激素加速的衰老过程得以延缓, 这与内源 ABA、MeJA、ZR 和 IAA 的含量变化有关, 而与 GA_3的含量变化无关。同时, ZR 和 IAA 的变化模式也说明了这两种激素的变化可能是缺失 PLDα1 延缓激素加速的衰老过程这一事件的原因而非结果。

关键词: 叶片衰老; 采后生理; 激素; 磷脂酶 Dα1; 拟南芥
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Analysis of Endogenous Hormone Levels to Reveal the Retardation by Suppression of Phospholipase Dα1 in *Arabidopsis* Leaves during Hormone-promoted Senescence

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Abstract: Postharvest senescence is affected greatly by endogenous and exogenous hormones. The promotive effects of exogenous abscisic acid (ABA) and ethylene on senescence are retarded by suppression of phospholipase Dα1 (PLDα1) in detached *Arabidopsis* leaves. However, understanding about the roles of endogenous hormones in the retardation of postharvest senescence by the suppression of PLDα1 activity remains incomplete. Here we report changes in the levels of endogenous ABA, methyl jasmonate (MeJA), indole-3-acetic acid (IAA), zeatin riboside (ZR), and gibberellic acid (GA_3) during leaf senescence induced by detachment and also accelerated by the application of exogenous ABA or ethylene in wild-type and PLDα1-deficient *Arabidopsis* leaves. The responses of the five hormones to the three treatments showed the existence of two stages during the artificial senescence, and, for each hormone, the pattern during the early stage was identical. The retardation of senescence in PLDα1-deficient plants was associ-

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ated with changes in ABA, MeJA, ZR, and IAA levels, but was independent of changes in endogenous levels of GA_3. The profiles of ZR and IAA suggest that changes in ZR and IAA levels might cause, and not simply be the consequence of, the retardation of senescence that is caused by suppression of PLDα1.

Key words: Leaf senescence; Postharvest physiology; Hormone; Phospholipase Dα1; *Arabidopsis thaliana*

Abbreviations: ABA, abscisic acid; CK, cytokinin; GA, gibberellic acid; MeJA, methyl jasmonate; JA, jasmonic acid; IAA, indole-3-acetic acid; PA, phosphatidic acid; PLD, phospholipase D; PS II, photosystem II; SAGs, senescence-associated genes; WT, wild-type

Leaf senescence is the final stage of leaf development when mobilisable nutrients, such as amino acid and lipids, are usually recycled (Lim *et al.*, 2007; Guiboileau *et al.*, 2010). Leaf senescence is a highly regulated process, and comprises a series of ordered events that occur at the physiological, biochemical, and molecular levels. Typical syndromes of senescence include yellowing of leaf margins, termination of photosynthesis, changes in the levels of endogenous hormones, degradation of membrane lipids, and the expression of senescence-associated genes (SAGs) (Smart, 1994; Thompson *et al.*, 1998; Ralph *et al.*, 2005). Leaf senescence can be induced by both developmental signals and environmental stimuli (Guo and Gan, 2005). The sudden disruption of their energy, nutrient, and hormone supplies causes harvested immature vegetables to enter into a senescent state (King *et al.*, 1990). Given that the degradative processes that occur in detached leaves show many similarities to the events that occur during natural leaf senescence, detached leaves are widely used as a model system to study leaf senescence (Page *et al.*, 2001).

The exogenous application of hormones can influence the progress of leaf senescence that is induced by detachment (Weaver *et al.*, 1998). For instance, the application of abscisic acid (ABA) or ethylene strongly promotes senescence in detached leaves, and in *Arabidopsis*, this process can be retarded by the suppression of phospholipase Dα1 (PLDα1) and PLDδ (Fan *et al.*, 1997; Jia *et al.*, 2013). PLD hydrolyzes phospholipids to yield phosphatidic acid (PA) and a soluble head group, and thus plays critical roles in the metabolism of membrane phospholipids and lipid signaling (Wang, 2005). As one of the most abundant members of the PLD family in *Arabidopsis*, PLDα1 regulates plant responses to abiotic stresses, such as drought, freezing, high salinity, and wounding, through influencing the signaling and/or structural roles of its product PA (Bargmann and Munnik, 2006). It has been proposed that the mechanism by which PLDα1 promotes senescence centres around the ability of PLDα1-mediated PA to destabilize membranes and activate other lipid-degrading enzymes, thus causing a loss of both membrane integrity and functionality of membrane-associated proteins (Fan *et al.*, 1997). Recent reports indicate that PLDα1-mediated PA has important functions in ABA signaling through its interaction with ABI1 and G protein (Hong *et al.*, 2010), which provides a potential link between PLDα1 and ABA-promoted senescence. Nonetheless, the precise details of the mechanism (s) involved remain unclear.

The progress of leaf senescence is regulated by the levels of endogenous hormones (Manju *et al.*, 2001; Gan, 2010). In general, ethylene, ABA (Manzano *et al.*, 2006), and methyl jasmonate (MeJA) or its precursor jasmonic acid (JA) promote senescence, whereas cytokinins (CKs), auxin, and gibberellic acid (GA_3) retard senescence (Gan, 2010). In addition to molecular genetic approaches, studies of the correlations between the levels of endogenous and/or exogenously applied hormones and the progress of senescence have been used frequently to investigate the roles of hormones in senescence (Gan, 2010). Changes in endogenous hormone levels had been examined in some plant species in detachment-induced senescence. For ex-

ample, changes in the levels of endogenous ABA were measured during the senescence of detached lettuce leaves (Aharoni and Richmond, 1978), tobacco leaves (Even-Chen and Itai, 1975), and nasturtium leaves (Chin and Beevers, 1970), whereas changes in the levels of endogenous auxin were measured in tobacco leaves (Evenchen *et al.*, 1978) and excised bean leaves (Sheldrak and Northcot, 1968). However, the correlations between hormone levels and senescence appear to differ among plant species (Sheldrak and Northcot, 1968). These differences underscore the need for improved understanding of the response of endogenous hormone levels to detachment-induced leaf senescence in *Arabidopsis*. The correlation between hormone levels and senescence remains undocumented in relation to leaf senescence that is promoted by ABA or ethylene. Furthermore, it remains to be established whether and how retardation of ABA- and ethylene-promoted senescence involves changes in endogenous hormone levels in the leaves of *Arabidopsis* plants with attenuated PLDα1 activity. Investigation of these correlations is of biological significance, given that the effects of PLDα1 on hormone synthesis are unclear so far.

To address the above-mentioned questions, we used enzyme-linked immunosorbent assays (ELISAs) to examine changes in the endogenous concentrations of MeJA, ABA, GA_3, and a representative CK (Zeatin riboside, ZR), and auxin (indole-3-acetic acid, IAA) during detachment-induced leaf senescence and ABA- and ethylene-promoted leaf senescence. We analyzed both wild-type (WT) *Arabidopsis* plants and transgenic plants in which expression of PLDα1 was attenuated using antisense-mediated suppression. Here we report changes in the patterns of endogenous accumulation of MeJA, ABA, GA_3, ZR, and IAA and the association of these hormone compounds with PLDα1-mediated retardation of ABA- and ethylene-promoted senescence. The patterns reveal the presence of two physiological stages of senescence in detached leaves.

1 Materials and methods

1.1 Plant materials, growth condition, wounding treatments, and hormone treatments

Arabidopsis thaliana, ecotype Columbia (wild-type, WT) and PLDα1-antisense (PLDα1-AS), which was generated from the Columbia ecotype and in which PLDα1 is constitutively attenuated by antisense-mediated suppression (Fan *et al.*, 1997), were used. The plants were grown in a controlled growth chamber at 23 ℃ (day) and 19 ℃ (night) and 60% relative humidity under a 12 h photoperiod with fluorescent lighting of intensity 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For wounding treatments, we followed the procedures described previously (Gepstein and Thimann, 1981) with minor modification. Leaves were wounded with a hemostat three times immediately after detachment and then incubated in water. The leaves were sampled at the indicated times and then stored in liquid nitrogen before MeJA extraction. The procedures that were used for phytohormone-induced senescence were described previously (Oh *et al.*, 1996). In brief, fully expanded leaves of the same age were collected from ~6-week-old plants, and the leaves were floated on water that contained either 50 $\mu mol \cdot L^{-1}$ ABA (Sigma, A1049) or 50 $\mu mol \cdot L^{-1}$ ethephon (Sigma, C0143), or on water without any hormone (Fan *et al.*, 1997) under the normal growth conditions. Ethephon is a water-soluble compound that releases ethylene in the cell, which enables researchers to evaluate the effects of ethylene more conveniently than treatments that involve the administration of ethylene gas.

1.2 Measurements of photosynthetic activity and cell death

The emission of chlorophyll fluorescence from the upper surface of the leaves was measured using an imaging chlorophyll fluorometer, MAXI-Imaging Pulse-Amplitude (PAM) (Walz, Germany), as described previously (Yang *et al.*, 2012). The maximal photochemical quantum yield of photosystem II (PS II) was determined in dark-adapted (20 minutes) samples on the basis of the initial level of fluorescence (F_0) and the maximal level of fluores-

cence (F_m), and calculated as $F_v/F_m = (F_m-F_0)/F_m$ and variable fluorescence (F_v).

Cell death was quantified spectrophotometrically by Evan's blue staining of detached leaves as described previously with minor modifications (Rea *et al.*, 2004). In brief, detached leaves were incubated with 0.1% Evan's blue for 2 h with shaking, and then washed extensively to remove unbound dye. The leaves were ground into powder in liquid nitrogen. The tissue powder was incubated with 50% methanol and 1% SDS at 60 ℃ for 30 min and then centrifuged. As a control for complete cell death, the leaves were heated at 100 ℃ for 5 min. The absorbance of the supernatant solution was measured at 600 nm.

1.3 Measurement of endogenous hormone levels

Plant tissue was extracted and samples were prepared using a slight modification of a previously described method (Lei *et al.*, 2007). Immediately after sampling, detached leaves (0.5 ~ 1 g) were homogenized in 2 mL of 80% methanol that contained 1 μmol·L^{-1} butylated hydroxytoluene and the contents were transferred to a separate container; the procedure was repeated twice. Rinsing the mortar in this way ensured that all plant material was included in the extract used for analysis. After centrifugation, the extracts were dried under nitrogen gas. The levels of endogenous hormones in the leaves were determined using ELISA kits that were obtained from Professor Baomin Wang (Zhao *et al.*, 2006; Deng *et al.*, 2008; Zhao *et al.*, 2011). Five replicates from each sampling time were analyzed. The data were subjected to one-way analysis of variance (ANOVA) with SPSS 16.0. Statistical significance was tested by Fisher's least significant difference (LSD) method.

2 Results

2.1 MeJA levels increased in an identical manner during detachment-induced senescence in WT and PLDα1-AS leaves

As indicated in Figure 1, we compared changes in F_v/F_m values (which indicate the photochemical quantum efficiency of PS II) and cell death between WT and PLDα1-AS plants during three treatments that induced senescence artificially. In the first treatment, detachment-induced senescence, detached *Arabidopsis* leaves were floated on water. In the second and the third, detachment-induced senescence was accelerated by the addition of ABA and ethephon, respectively. A visible sign of leaf yellowing and a decrease in F_v/F_m values was characterized during the three senescence treatments. In the absence of ABA or ethephon, detached leaves stayed green after 5 days, leaves detached from WT plants started yellowing 1 day after treatment and most parts turned yellow 5 days after incubation in 50 μmol·L^{-1} ABA and ethephon. In contrast, most parts of the PLDα1-suppressed leaves were still green after the 5-day hormone treatment (Fig. 1A). Consistent with the decreased loss of chlorophyll, the PLDα1-suppressed leaves retained a higher photosynthetic activity than that of WT leaves (Fig. 1A). After a 5-day treatment with ABA and ethephon, the cell death rate in WT leaves were 25, 62 and 60% of their respective initial values, which obviously higher than their corresponding control PLDα1 antisense leaves (Fig. 1B).

We detached leaves from intact plants by cutting the leafstalk. These leaves not only experience senescence but are also subject to mechanical wounding. To test the potential effects of wounding, we first examined levels of MeJA in the detached leaves that were floated on water. Wounding usually causes transient increases in levels of MeJA within 3 hours, and this increase is suppressed in PLDα1-AS *Arabidopsis* plants (Wang *et al.*, 2000). In our control experiments in which leaves were wounded by hemostat twice (Fig. 2A), the MeJA levels in WT leaves increased to 234%, 174% and 187% of its initial levels at day 1, 2 and 3, respectively. MeJA levels were significantly higher in WT leaves than in PLDα1-AS leaves after wounding for 1 and 2 days. (Fig. 2A). In contrast, in our detachment experiments in which leave were cut at leafstalk (Fig. 2B,

top panel), the levels of MeJA increased 188% at day 1 and then went back to the initial levels. The extensity and duration of the effects in detachments was much less than that in wounding. Most importantly, no difference of MeJA levels between WT and PLDα1-AS plants was observed after leaves were cut. This indicates that the wounding effects from cutting leafsalk was small and could be ignored.

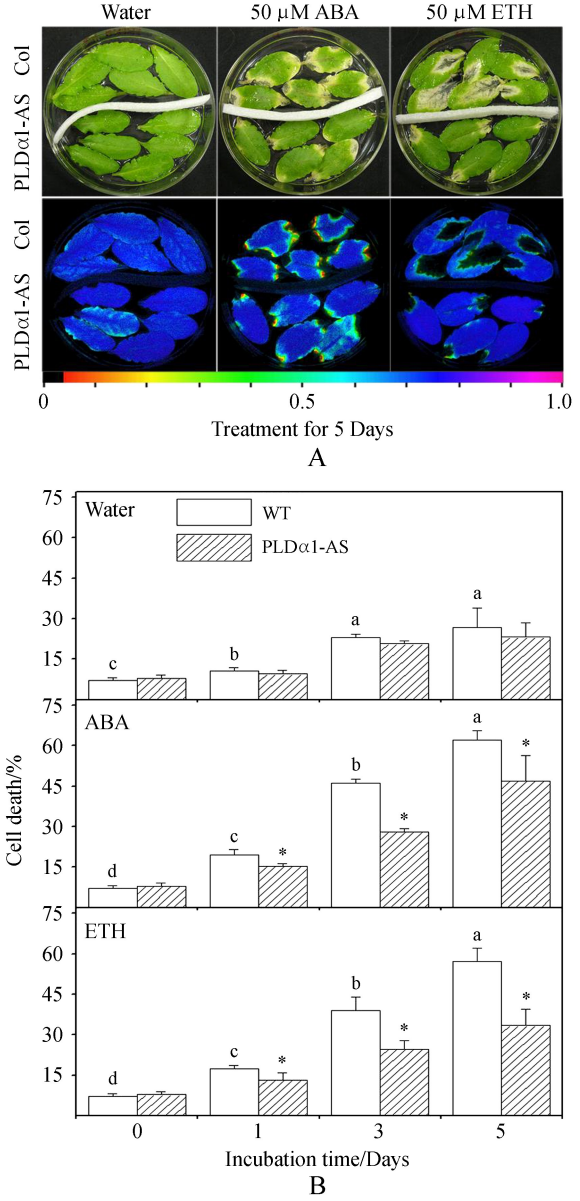

Fig. 1　Senescence of detached leaves from WT and PLDα1-AS *Arabidopsis* plants

Notes: Leaves that had been detached from WT and PLDα1-AS plants were treated with sterile water, 50 μmol · L^{-1} ABA, or 50 μmol · L^{-1} ethephon (ETH) for 5 days. A. Retardation of ABA- and ethylene-promoted senescence in detached leaves of PLDα1-AS as compared with WT plants. Yellowing of the leaves (top panel) or low F_v/F_m values for variable fluorescence (bottom panel) indicated the presence of senescence. B. Cell death was determined spectrophotometrically using Evan's blue staining. Values are the mean ± *SD* ($n=5$)

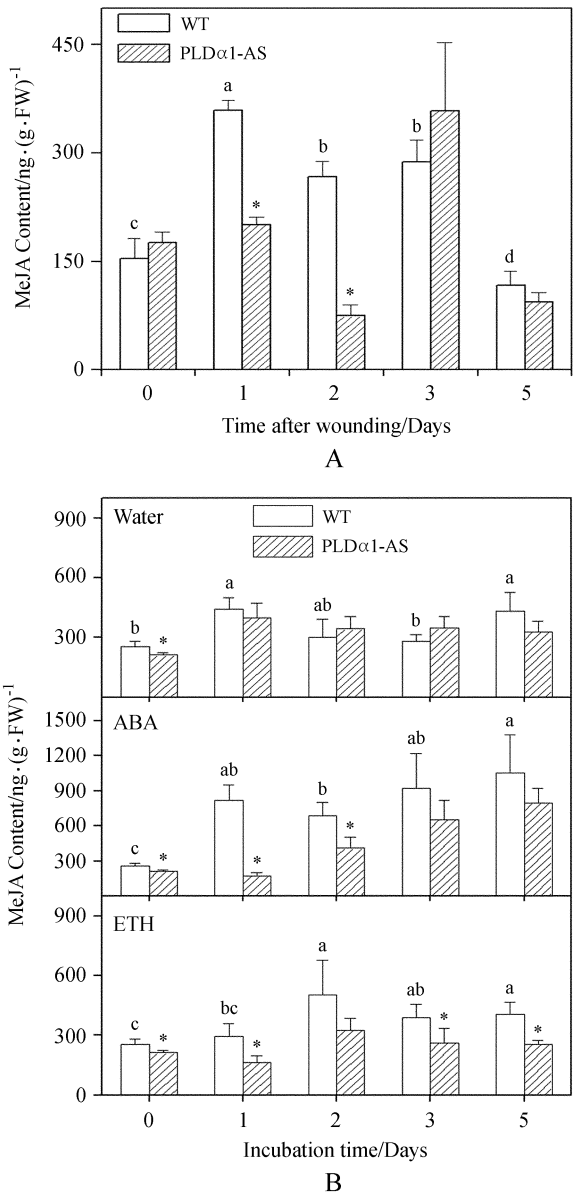

Fig. 2　Change in levels of endogenous MeJA in leaves from WT and PLDα1-AS plants (a) after mechanical wounding and (b) during three different senescence treatments

Notes: A. Leaves were treated with sterile water after wounding. B. Detached leaves were treated with sterile water (top), 50 μmol · L^{-1} ABA (middle), or 50 μmol · L^{-1} ethephon (ETH; bottom) and sampled at the indicated time points. FW, fresh weight. Values are the mean ± *SD* ($n=5$). Values with different letters are significantly different ($P<0.05$). "*" indicates that the value is significantly different from that of the WT under the same condition ($P<0.05$)

We thus ignored the wounding effects as a result of the procedures used in our study.

2.2 An increase in MeJA levels during hormone-promoted senescence is attenuated by suppression of PLDα1

The hormone MeJA and its precursor JA play critical roles in the regulation of senescence. In the present study, both the ABA and ethylene treatments increased the levels of MeJA in detached WT *Arabidopsis* leaves (Fig. 2B, middle and bottom panels). However, MeJA levels were significantly lower in PLDα1-AS leaves than in WT leaves (Fig. 2B, middle and bottom panels). These effects differed in terms of intensity between the ABA and ethylene treatments. Both the increase in MeJA and the inhibition of the increase in MeJA by the suppression of PLDα1 were larger under ABA treatment than under ethylene treatment. These results indicate that changes in MeJA levels are associated with the retardation of hormone-promoted senescence, in particular ABA-promoted senescence, that is mediated by the suppression of PLDα1 activity.

2.3 ABA levels increase at the onset of senescence and decline during the late stage of senescence

We examined the changes in endogenous ABA levels that occurred during the three senescence treatments mentioned above. In WT *Arabidopsis* leaves, ABA levels increased significantly during the early stage of senescence and then decreased during the late stage (Fig. 3, top panel). The levels of ABA in detached leaves that were incubated in water increased 1.5-fold within 2 days and then began to decrease over subsequent days, eventually dropping to 50% of the initial level after treatment for 5 days (Fig. 3, top panel). The sudden large increase in endogenous ABA after the exogenous application of ABA (Fig. 3, middle panel) might have been caused by the diffusion and/or uptake of ABA from outside. Nonetheless, even though the level of ABA increased to its highest point within 1 day of the start of ABA treatment, it then began to decrease. Within 1 day after ethylene treatment, ABA levels increased 1.7-fold, and then began to decrease, eventually reaching 25% of the initial level by day 5 (Fig. 3, bottom panel). These results indicated that transient increases in the levels of endogenous ABA are associated with the triggering of senescence, and that, at later stages of senescence, ABA levels decreased in leaves treated with exogenous ABA or ethylene.

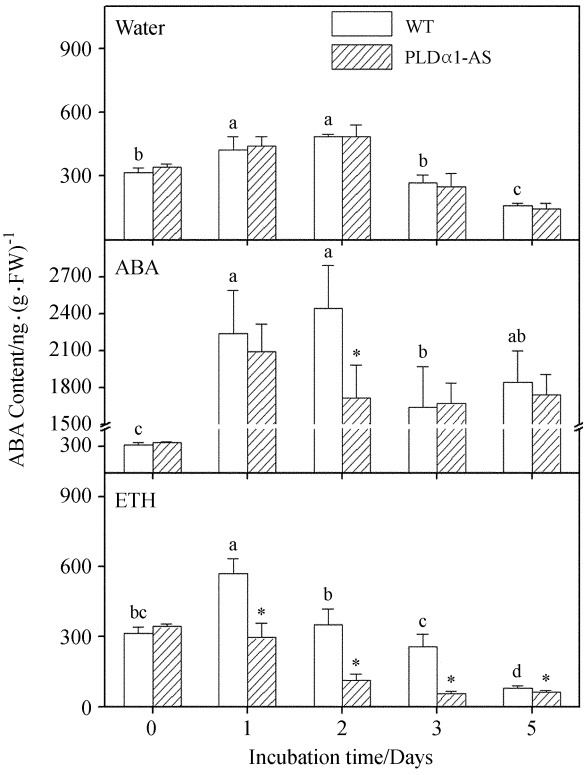

Fig. 3 Change in levels of endogenous ABA in leaves from WT and PLDα1-AS plants during three different senescence treatments

Notes: Detached leaves were treated with sterile water (top), 50 μmol·L^{-1} ABA (middle), or 50 μmol·L^{-1} ethephon (ETH; bottom) and sampled at the indicated time points. FW, fresh weight. Values are the mean ± *SD* ($n=5$). Values with different letters are significantly different ($P<0.05$). "*" indicates that the value is significantly different from that of the WT under the same condition ($P<0.05$)

2.4 Suppression of PLDα1 decreases levels of endogenous ABA during ethylene-accelerated senescence

To further investigate the roles of ABA in senescence, we analyzed ABA levels in PLDα1-AS plants, in which hormone-accelerated senescence was delayed relative to that in WT plants (Fig. 1). To avoid the possible complication that levels of en-

dogenous ABA are affected by the uptake of exogenously applied ABA during ABA-induced senescence, we focused on the levels of endogenous ABA in detached leaves under water and ethylene treatments (Fig. 3). Levels of ABA were identical between WT and PLDα1-AS plants during detachment-induced senescence (Fig. 3, top panels). However, there were significant differences in ABA levels between WT and PLDα1-AS plants during ethylene-induced senescence. No transient increase in ABA levels occurred during the early stage of senescence in PLDα1-AS leaves, and ABA levels were significantly lower in PLDα1-AS leaves than in WT leaves (Fig. 3, bottom panel). The data indicated that the delayed senescence that was associated with the suppression of PLDα1 correlated with a decrease in ABA levels, which meant that the levels of endogenous ABA contributed to this delay in ethylene-promoted senescence.

2.5 ZR levels decrease during detachment-induced senescence and suppression of PLDα1 attenuates the decline of ZR abundance during hormone-accelerated senescence

We measured changes in the content of ZR, the major bioactive cytokinin, during the three senescence treatments defined above. During detachment-induced senescence (Fig. 4, top panel), ZR levels dropped to 30% of their initial levels within 1 day after detachment and then kept decreasing until they reached 10% of their initial levels at day 5. We found no differences in ZR levels between leaves from WT and PLDα1-AS plants during detachment-induced senescence, except that ZR levels were slightly lower in PLDα1-AS than in WT leaves at day 2. However, during ABA- and ethylene-accelerated senescence (Fig. 4, middle and bottom panels), the decreases in ZR levels were less dramatic and were subject to greater fluctuations over the course of the experiments. This indicates that senescence in detached leaves is associated with a decrease in the abundance of ZR. In addition, the pattern of fluctuation in ZR levels was distinct between ABA- and ethylene treatments. Moreover, the levels of ZR in PLDα1-AS leaves were significantly higher than those in WT leaves during the late stage of ABA treatment, when ZR reached the highest levels, and during the early stage of ethylene treatment, when ZR remained at the highest levels. These results suggest that the changes in the levels of endogenous ZR, or cytokinin-like compounds, are not the consequence of hormone-promoted senescence but contribute to the regulation of the attenuation of hormone-accelerated senescence in PLDα1-AS leaves. Results also indicated that PLDα1-AS leaves respond differently to ABA and ethylene-induced senescence.

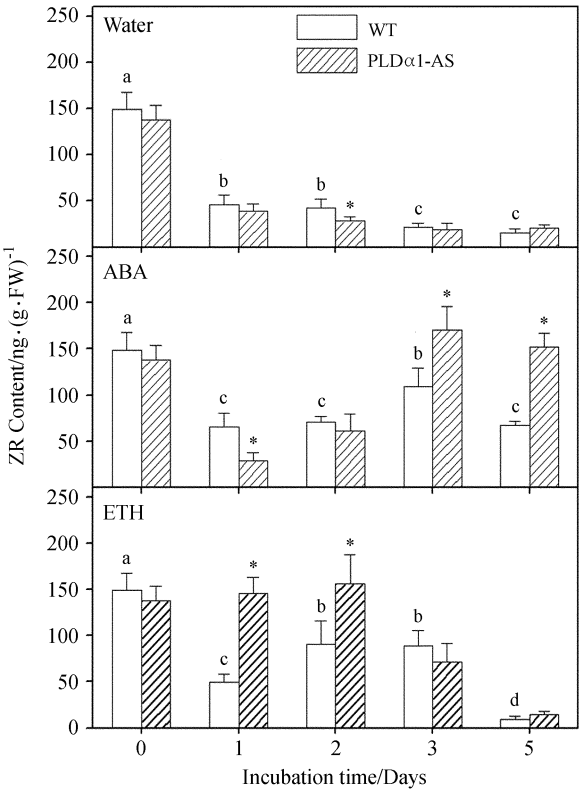

Fig. 4　Chnage in levels of endogenous ZR in leaves from WT and PLDα1-AS plants during three different senescence treatments
Notes: as the same as the fig. 3

2.6 IAA levels decrease transiently during the early stages of both detachment-induced and hormone-accelerated senescence, and are higher in PLDα1-AS leaves than in WT leaves

We analyzed the changes that occurred in the levels of IAA, a well-known form of auxin, during

the three senescence treatments defined above. The concentration of IAA decreased dramatically on the first day in both water- and hormone-treated detached leaves, and then returned to almost the initial levels during the later stage (Fig. 5). The decrease at the onset of detachment-induced senescence may be the consequence of the sudden disruption of nutrient and energy supplies. During ABA-accelerated senescence, levels of IAA were slightly, but significantly, higher in PLDα1-AS leaves than in WT leaves at day 5, whereas no obvious differences were detected at other sample time. During ethylene-accelerated senescence, levels of IAA in PLDα1-AS leaves was 1.6-fold, 1.5-fold and 1.2-fold compared to WT leaves after treatment for 1, 2, and 3 days, respectively (Fig. 5, middle and bottom panels). These results indicated that attenuation of hormone-accelerated senescence in PLDα1-AS plants was associated with an increase in IAA content. The results also suggested that changes in the levels of endogenous auxin, at least auxin-like compounds, might not just be the consequence of, but also be involved in, the regulation of ABA- and ethylene-accelerated senescence.

2.7 Gibberellic acid (GA_3) levels decrease during detachment-induced senescence but do not change during ABA- and ethylene-accelerated senescence

In the present study, levels of GA_3 decreased during detachment-induced senescence (Fig. 6, top panel). However, the levels of GA_3 during ABA- and ethylene-accelerated senescence were no significant difference to those in the control (Fig. 6, middle and bottom panels). There were no differences in GA_3 levels between WT and PLDα1-AS plants under any of the conditions. The results showed that levels of GA_3, or giberellin-like compounds, are negatively correlated with the progression of detachment-induced senescence, but do not contribute to hormone-accelerated senescence.

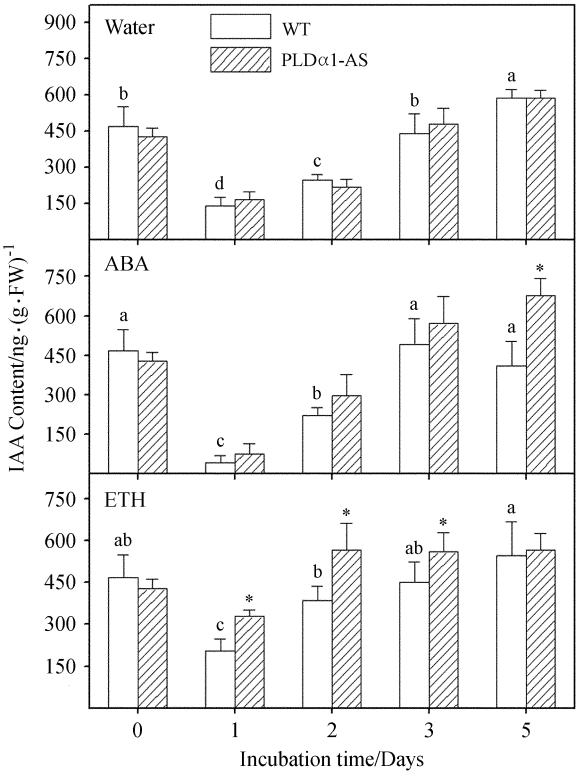

Fig. 5 Change in levels of IAA in leaves from WT and PLDα1-AS plants during three different senescence treatments

Notes: as the same as the fig. 3

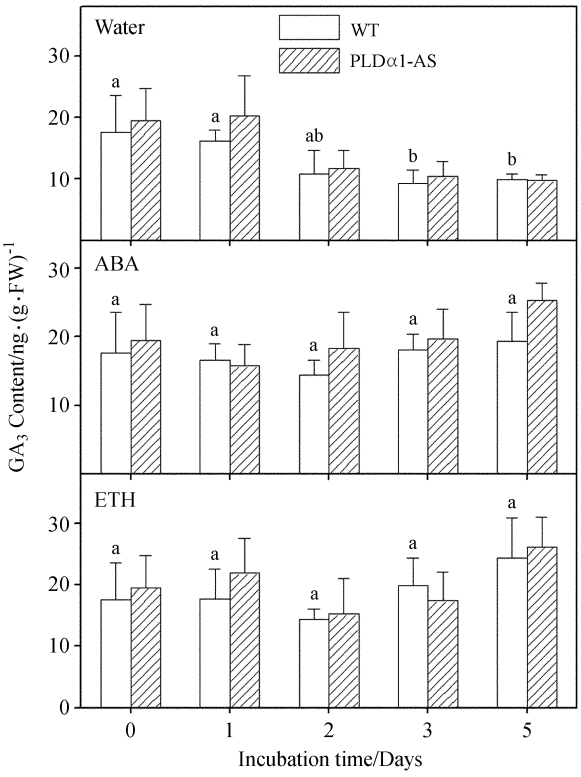

Fig. 6 Change in levels of endogenous GA_3 in leaves from WT and PLDα1-AS plants during three different senescence treatments

Notes: as the same as the fig. 3

3 Discussion

An understanding of postharvest senescence, and the mechanisms that can retard the process, is very important in relation to efforts to enhance crop productivity (Page *et al.*, 2001; Guo and Gan, 2005). Hormones play critical roles in these processes, although much remains to be learned about correlations between hormone levels and senescence, particularly during hormone-promoted senescence. The effects of hormones on the retardation of senescence in PLDα1-deficient plants have never been reported. After confirming that mechanical wounding by cutting the leaf stalk did not cause substantial effects in detached *Arabidopsis* leaves, we used a detached-leaf system to examine the endogenous levels of five commonly studied hormones during three senescence treatments. This approach enabled us to monitor correlations between changes in the patterns of the hormone levels and the progress of senescence in leaves from both WT and PLDα1-AS *Arabidopsis* plants. The patterns and differences that emerged from our analysis are summarized in Table 1 and Table 2, respectively. In addition to enabling us to compare and contrast the roles of hormones in detachment-, ABA-, and ethylene-promoted senescence and providing insights into the role of PLDα1 in senescence, these comprehensive data both support previous data and reveal new insights into the roles of plant hormone levels during senescence. For example, the observed changes in the levels of endogenous MeJA, ABA, and IAA during detachment-induced leaf senescence in *Arabidopsis* (Fig. 2, 3, and 5) were similar to those reported for other species (Gepstein and Thimann, 1980; He *et al.*, 2002; Van der Graaff *et al.*, 2006; Ghanem *et al.*, 2008). Here, we have expanded on these studies by reporting that levels of ZR decreased during the whole course of detachment-induced senescence. ZR levels decreased, and then sightly increased eventually dropped again during the whole course of ABA- or ethylene-promoted senescence (Table 1). Meanwhile, levels of GA_3 remained unchanged throughout all three conditions, except during the later stage of detachment-induced senescence, when GA_3 levels decreased. Leaf senescence caused by detachment resulted in the dropping in CK levels (Fig. 4) which was believed to be a key signal for the senescence initiation (Nooden *et al.*, 1990) In addition, acceleration of senescence by ABA and ethephon involved a increase of ZR levels rather than keeping dropping as it did in leaves just incubated in water (Fig. 2A). Analysis of cytokinin levels in leaves before and after the onset of senescence has revealed an inverse correlation between cytokinin levels and the progression of senescence in a variety of tissues and plant species (King *et al.*, 1990; Gan and Amasino, 1995). The increase of ZR levels was examined in the progression of ABA- and ethephon-accelerated senescence might due to the interaction took place between external application hormones with endogenous ZR. The elevation of endogenous ZR levels might be in order to antagonize the senescence-promoting effect of ABA and ethephon.

Table 1　Patterns of changes in hormone levels in detached WT *Arabidopsis* leaves during three senescence treatments: water, ABA, and ethephon, respectively

Hormone	Change in endogenous levels in WT		
	Detachment	ABA	Ethylene
ABA	↗↘	*	↗↘
JA	↗→	↗→	↗→
ZR	↘→	↘→	↘→
IAA	↘↗	↘↗	↘↗
GA_3	→↘	→→	→→
ZR	↘↘	↘↗↘	↘↗↘

"↗" indicates an increase in hormone levels, "→" indicates a maintenance of hormone levels, and "↘" indicates a decrease in hormone levels. "*" indicates that the observed effect might be an artifact caused by interference owing to the presence of exogenous ABA

Senescence of intact leaves can be divided conveniently into two stages, on the basis of their reversibility. Whereas the first stage of senescence is reversible, the second stage, which involves cell death and even necrosis, is not (Buchanan-Wollaston *et al.*, 2003). For detachment-induced senes-

cence, our data indicated that there are two stages that are characterized by different trends in the ways in which hormone levels change (Table 1). For example, whereas ABA levels increase first before declining, levels of IAA decrease first and then increase. These two stages may serve distinct physiological roles. As shown in Table 1, during the first stage, the response of each of the five hormones did not differ among the three treatments during the artificial senescence: regardless of whether senescence was induced by detachment, or accelerated by either ABA or ethephon, levels of ABA and MeJA always increased, levels of ZR and IAA always decreased, and levels of GA_3 remained unchanged. These data suggest that the changes in hormone levels that occur during the first stage of all three treatments are responses to common factors that mediate their effects shortly after detachment. Given that the effects of mechanical wounding on hormone levels of detached leaves is small and can be ignored, as already discussed, such factors might be related to the disruption of energy and nutrient supplies. During the later stage, there were some differences in the responses of the five hormones to the three treatments during senescence. Levels of GA_3 decreased during leaf segments kept in water, but remained unchanged in segments kept in ABA or ethephon (Table 1). Therefore, we propose that, during the initial stage, the observed changes in hormone levels are responses to deficits in energy and nutrient supplies, whereas during the later stage, they are responses to hormone-mediated effects.

It has been proposed that suppression of PLDα1 activity retards hormone-promoted senescence by interfering with structural roles of PA, which is largely produced by PLDα1 (Fan *et al.*, 1997). However, recent progress has revealed that PLDα1 and PA also participate in hormone signalling, including the transduction of signals that are downstream of the perception of ABA and JA (Gepstein and Thimann, 1981; Ritchie and Gilroy, 1998; Wang *et al.*, 2000). Therefore, the effects of PLDα1 on senescence might be mediated, at least in part, through the actions of hormones. The results of the present study revealed no differences in either phenotypes or hormone levels between the leaves of WT and PLDα1-AS plants during detachment-induced senescence. However, during hormone-promoted senescence, we noted significant differences between the leaves of WT and PLDα1-AS plants in relation to both phenotypes and levels of MeJA, ZR, and IAA (Fig. 1 and Table 2). These results indicate a significant relationship between the retardation of senescence following antisense-mediated suppression of PLDα1 activity and changes in the levels of endogenous MeJA, ZR, and IAA. Levels of GA_3 do not appear to participate in these treatments.

Table 2 Differences in levels of hormones between WT and PLDα1-AS plants during three senescence treatments: water, ABA, and ethephon, respectively

Hormone	Difference in levels of hormones between WT and PLDα1-AS plants during senescence		
	Detachment	ABA	Ethylene
ABA	0	*	−
JA	0	−	−
ZR	0	− +	+ 0
IAA	0	0 +	+ 0
GA_3	0	0	0

The symbols "+", "0", and "−" indicate that the levels of hormones in PLDα1-AS plants are higher than, equal to, and lower than those in WT plants, respectively. "*" indicates that the observed effect might be an artifact caused by interference owing to the presence of exogenous ABA

It remains to be established whether changes in the levels of endogenous MeJA, ZR, and IAA are causal factors or just the consequences of the PLDα1-mediated retardation of hormone-promoted senescence. Membrane degradation is a major cellular process during leaf senescence (Thompson *et al.*, 1998). Considering the key roles of PLDα1 and its product PA in the metabolism of membrane lipids, one possibility is that the suppression of PLDα1 delays the degradation of membranes and this delay results in the retardation of senescence. In this case,

levels of MeJA, ZR, and IAA may be only the consequences of the retardation of senescence. Nonetheless, our data might provide another clue to answering this question, given that the differences in the levels of ZR and IAA between WT and PLDα1-plants during ABA treatment differed from those that occurred during ethylene treatment (Table 2). Thus, higher content of ZR and IAA in PLDα1-AS plants might contribute to its retardation of hormone-promoted senescence.

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